US 2006/0051371-A1

Pinol *et al*., Appl. No 10/535,416

"Live attenuated vaccine against porcine pleuropneumonia"

(HIPRA)

June 2009

APP bacteria

Apx exotoxins (members of RTX toxins family):

- ApxI: strong haemolytic and high immunogenic
  Operon *apxICABD* (*apxIC*, *apxIA*, *apxIB*, *apxID* genes)

- ApxII: weak haemolytic and low immunogenic
  Operon *apxIICAΔB* (*apxIIC*, *apxIIA*, *apxIIΔB* genes)

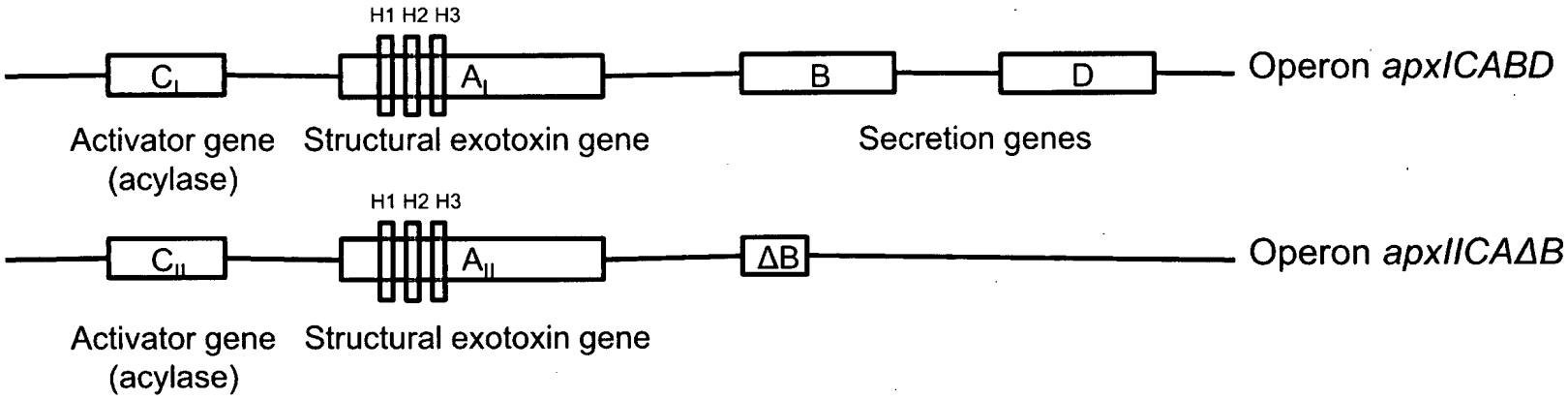
Genes:

- *apxIC*: activator gene for ApxI exotoxin
- *apxIA*: structural gene for ApxI exotoxin
- *apxIIC*: activator gene for ApxII exotoxin
- *apxIIA*: structural gene for ApxII exotoxin
- *apxIB* and *apxID*: secretion genes of ApxI and ApxII exotoxins
- *apxIIΔB*: non-operative fragment

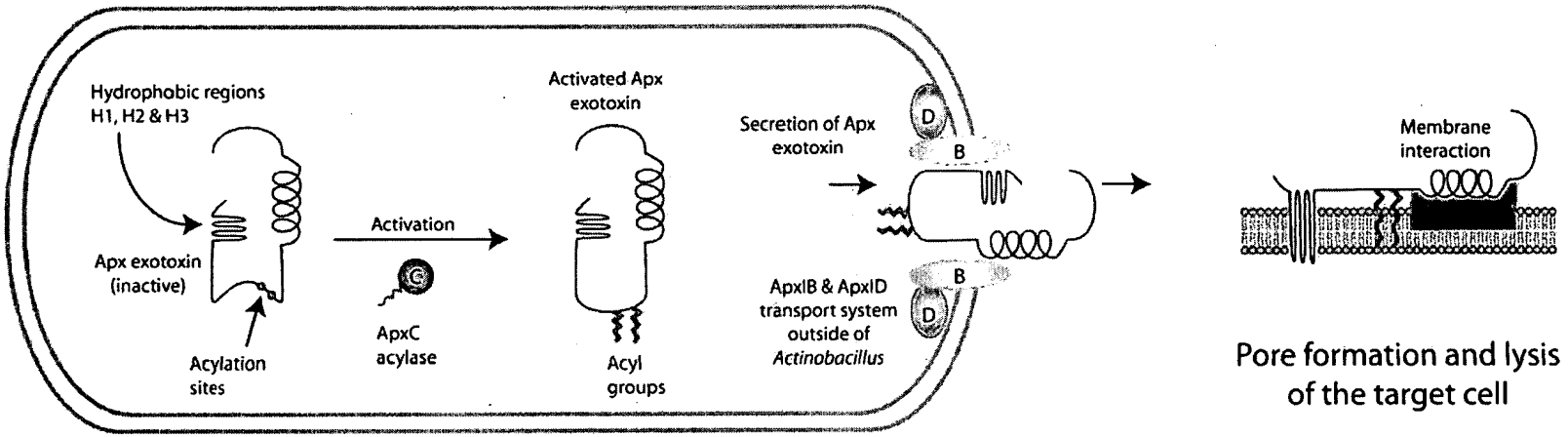
Apx exotoxins expression (several examples):

- Serotype 1: ApxI and ApxII exotoxins
- Serotype 10: only ApxI exotoxin
- Serotype 7: only ApxII exotoxin

Structure of genes codifying ApxIA and ApxIIA exotoxins

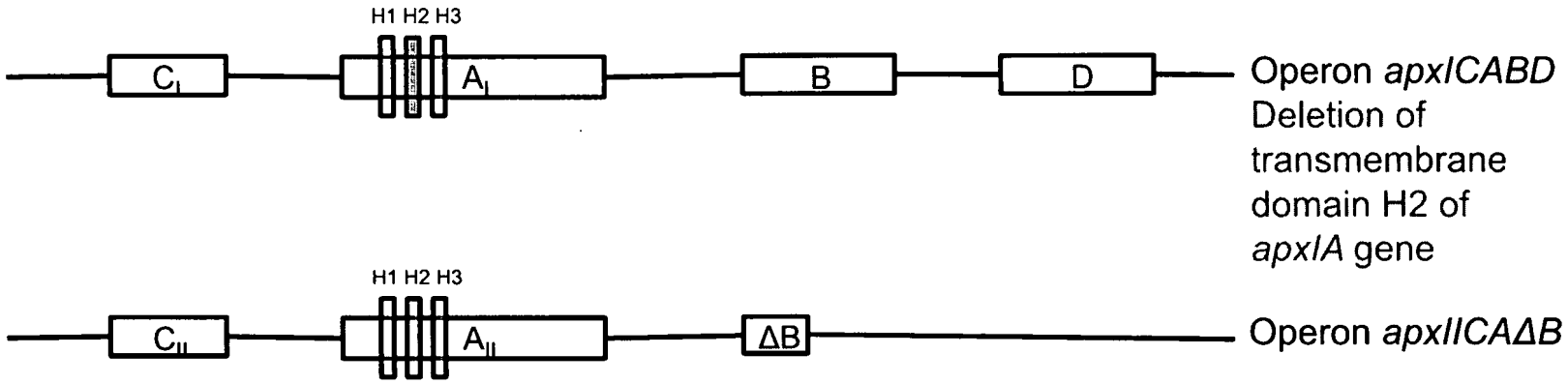


Expression, activation and secretion of Apx exotoxins



Pinol *et al.*, US 2006/0051371-A1

1) Deletion of a transmembrane domain of *apxIA* gen

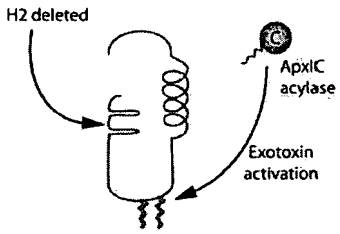


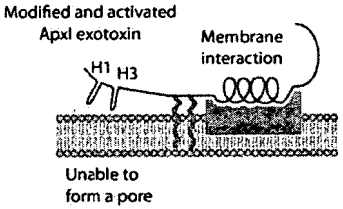
Production and secretion of activated, but no haemolytic ApxI exotoxin, and activated ApxII exotoxin

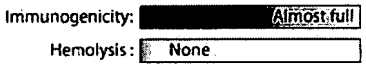
High immunogenic because ApxI and ApxII exotoxins are secreted

Weak haemolytic due to weak haemolytic activity of ApxII exotoxin (see Figure V)

FIGURE V
apxIAΔH2 + *apxIC* genes
(HIPRA 1)

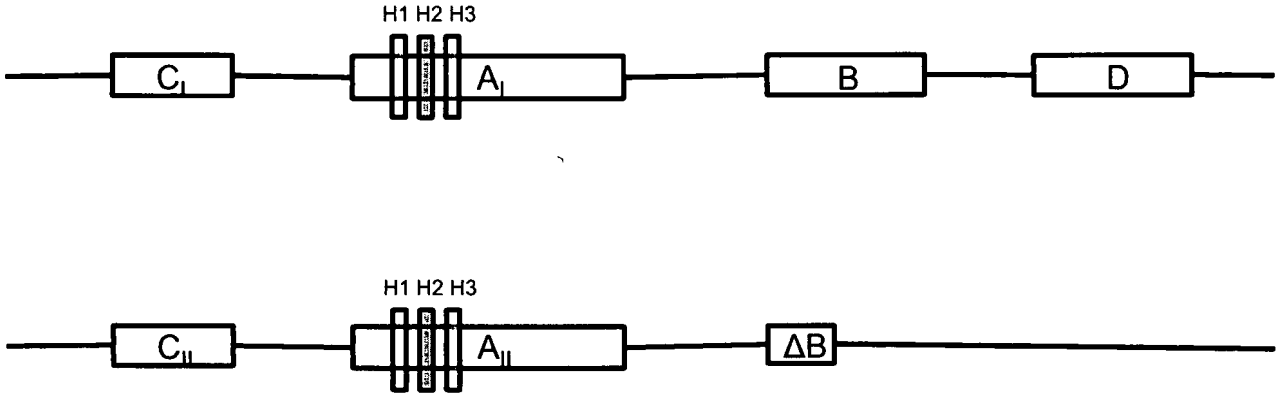






Pinol *et al.*, US 2006/0051371-A1

2) Deletion of a transmembrane domain of *apxIA* gene and of a transmembrane domain of *apxIIA* gene



Operon *apxICABD*
Deletion of a transmembrane domain H2 of *apxIA* gene

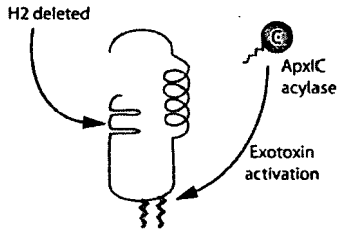
Operon *apxIICAΔB*
Deletion of a transmembrane domain H2 of *apxIIA* gene

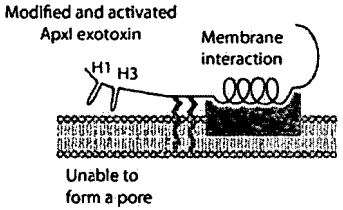
Production and secretion of activated, but no haemolytic, ApxI and ApxII exotoxins

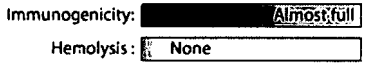
High immunogenic because ApxI and ApxII exotoxins are secreted

Non-haemolytic because modified ApxI and ApxII exotoxins are not capable to form pores (see Figure V)

FIGURE V
apxIAΔH2 + *apxIC* genes
(HIPRA 1)

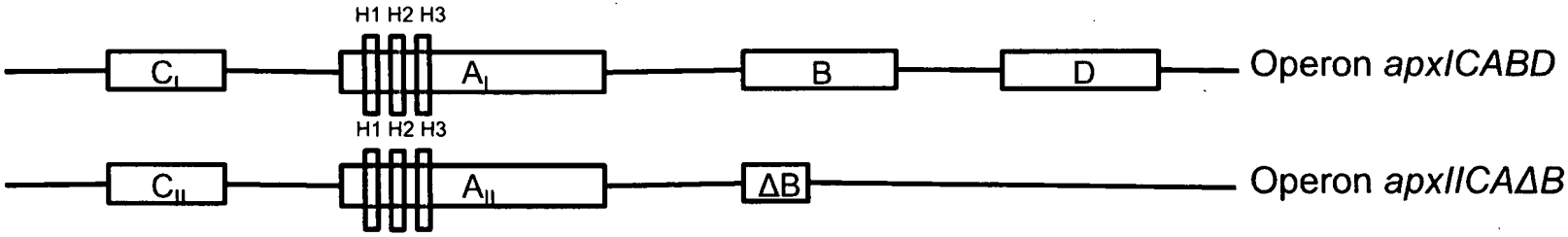






Reimer *et al.*, Microbial Pathogenesis, 1995, 18: 197-209

1) Strain J45: field isolate

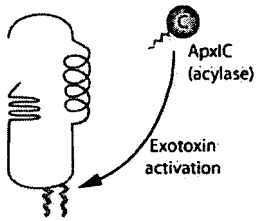


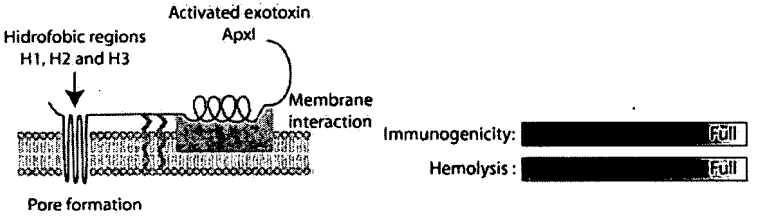
Production and secretion of activated ApxI and ApxII exotoxins

High immunogenic because it secretes ApxI and ApxII exotoxins

Strong haemolytic because ApxI and ApxII exotoxins are capable of forming pores
(see Figure I)

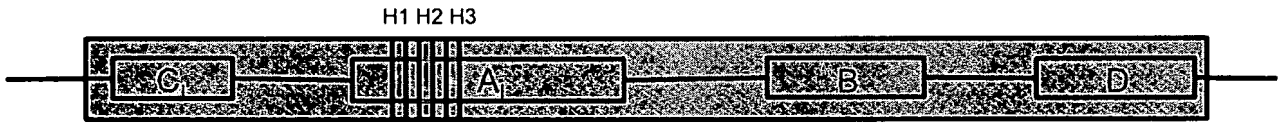
FIGURE I
apxIA and *apxIC* genes
(Reimer et al.)



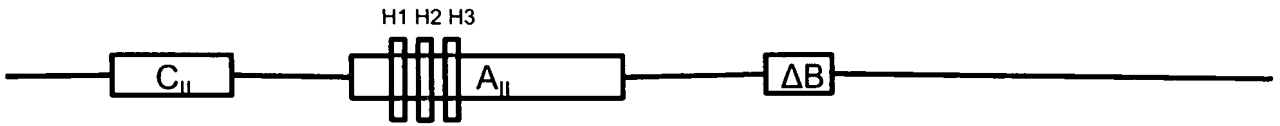


Reimer *et al.*, Microbial Pathogenesis, 1995, 18: 197-209

2) mIT4: chemical mutant



Operon *apxICABD*
apxIC, *apxIA*,
apxIB, *apxID*
genes completely
deleted



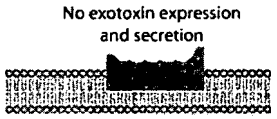
Operon *apxIICAΔB*

No production of ApxI exotoxin because of deletion of the whole *apxICABD* operon
Production but no secretion of activated ApxII exotoxin because of deletion of *apxIB* and *apxID* genes

Non-immunogenic because ApxI and ApxII exotoxins are not secreted

Non-haemolytic because ApxI and ApxII exotoxins are not secreted (see Figure II)

FIGURE II
ΔapxICABD genes
(Reimer et al.)

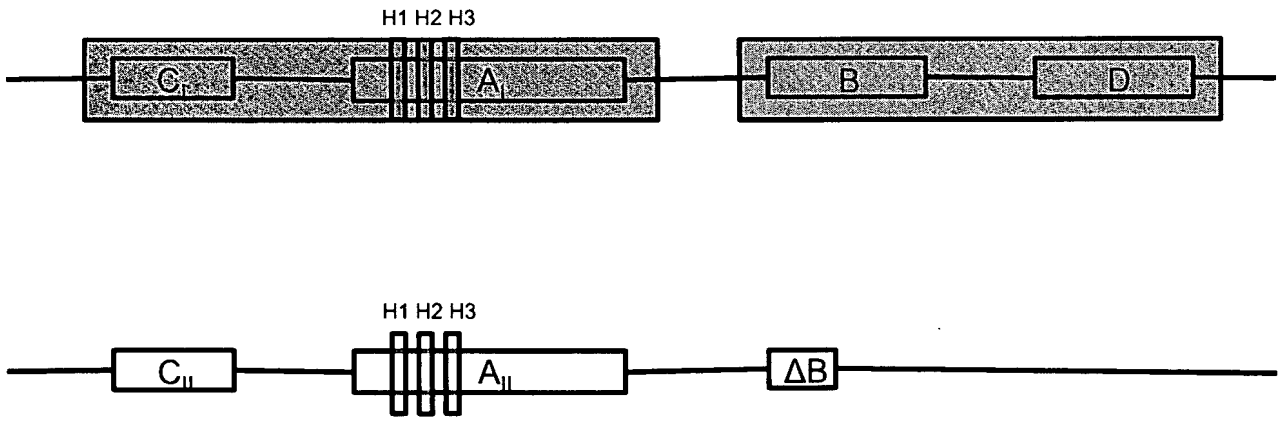


Immunogenicity: None

Hemolysis : None

Reimer *et al.*, Microbial Pathogenesis, 1995, 18: 197-209

3) Strain mIT4-H/pJFF801: chemical mutant with restored operon *apxIBD*



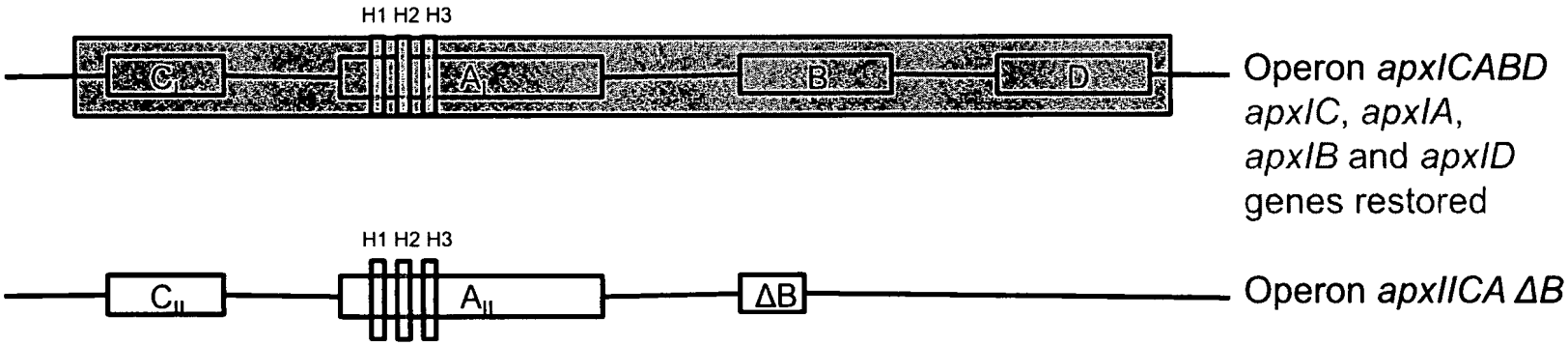
No production of activated ApxI exotoxin because a*pxIA and apxIC* genes are completely deleted
Production and secretion of activated ApxII exotoxin

Low immunogenic because ApxI exotoxin is not produced

Weak haemolytic because ApxII exotoxin is secreted

Reimer *et al.*, Microbial Pathogenesis, 1995, 18: 197-209

4) Strain mIT4-H/pJFF800: chemical mutant with restored operon *apxICABD*

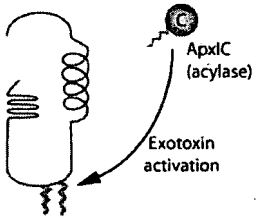


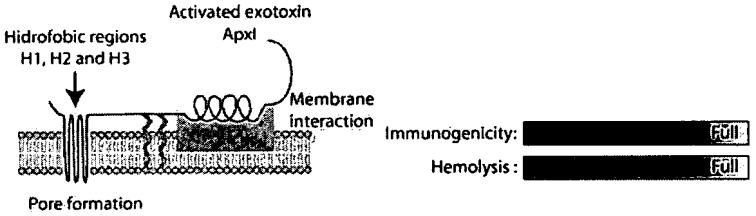
Production and secretion of activated ApxI and ApxII exotoxins

High immunogenic because it secretes ApxI and ApxII exotoxins

Strong haemolytic because ApxI and ApxII exotoxins are capable of forming pores
(see Figure I)

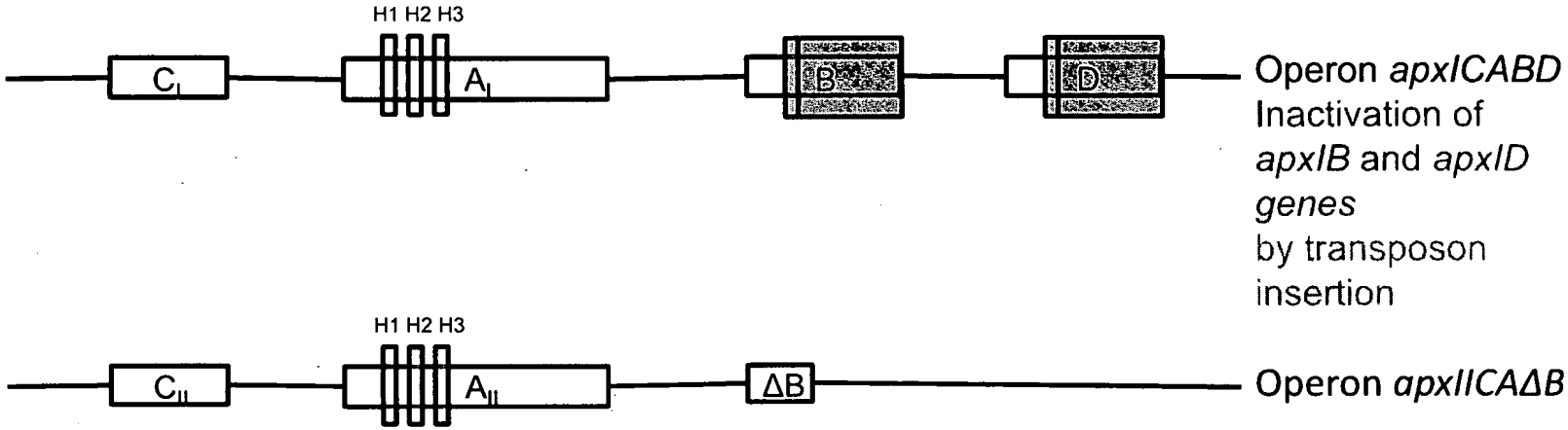
FIGURE I
apxIA and *apxIC* genes
(Reimer et al.)





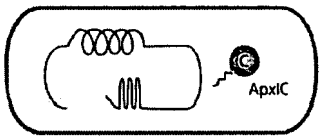
MacInnes *et al.*, US 6,019,984

Inactivation of *apxIB* and *apxID* genes (secretion genes) by transposon insertion (Example 5)



Production of cell-associated, activated ApxI and ApxII exotoxins, but they are not secreted
(see Figure III)

FIGURE III
apxIA and *apxIC* genes
Δ*apxIB* and Δ*apxID* genes
(MacInnes et al.)
(Prideaux et al.)



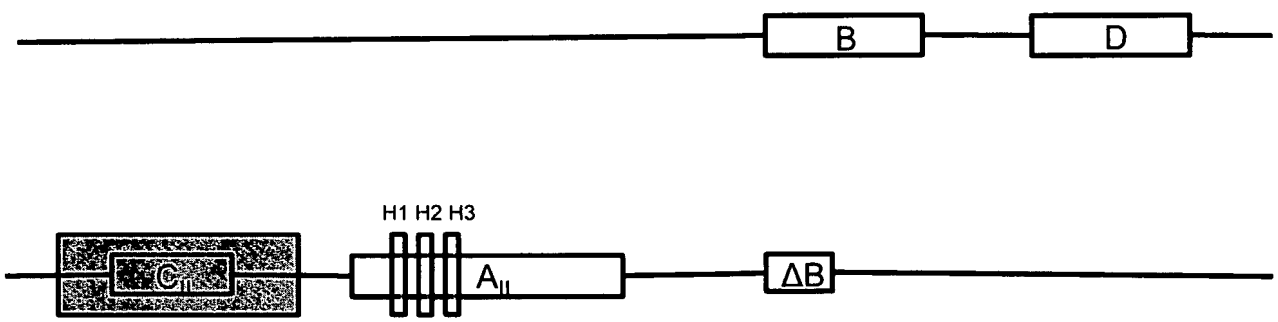
ApxIA is *not* secreted

No exotoxin secretion

Immunogenicity: None
Hemolysis: None

Prideaux *et al.*, US 6,472,183

1) Inactivation or deletion of *apxIIC* gene (activation gene) in wild strain HS93 (Serotype 7): strain HS93C$^-$
(Examples 10 and 11; column 20, lines 57-60; claims 1, 2, 3, 11, 12 and 14)

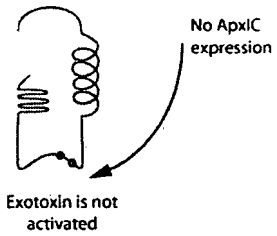


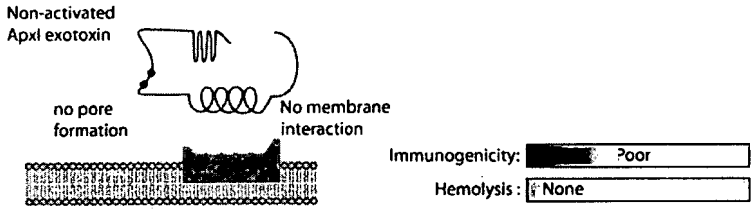
Operon *apxICABD*
apxIC and *apxIA* genes naturally inactivated

Operon *apxIICAΔB*
apxCII gene inactivated or deleted

Production and secretion of non-activated ApxII exotoxin
No production of ApxI exotoxin because of natural inactivation of *apxIC* and *apxIA* genes
(see Figure IV)

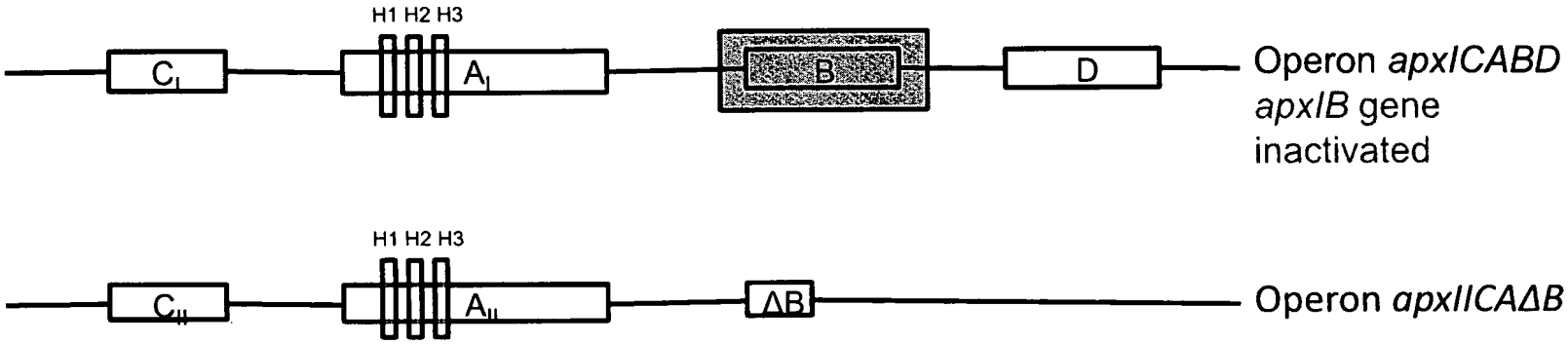
FIGURE IV
apxIA and not *apxIC* genes
or
apxIIA and Δ*apxIIC* genes
(Prideaux)





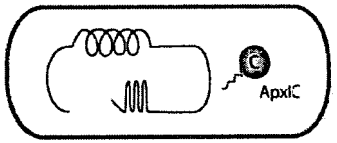
Prideaux *et al.*, US 6,472,183

2) Inactivation of *apxIB* gene (secretion gene) in wild strain HS22 (Serovar 1): strain HS22B$^-$
(Examples 9 and 11; column 5, lines 21-24)



Production but no secretion of activated ApxI and ApxII exotoxins , because of inactivation of *apxIB* gene
(see Figure III)

FIGURE III
apxIA and *apxIC* genes
Δ*apxIB* and Δ*apxID* genes
(MacInnes et al.)
(Prideaux et al.)



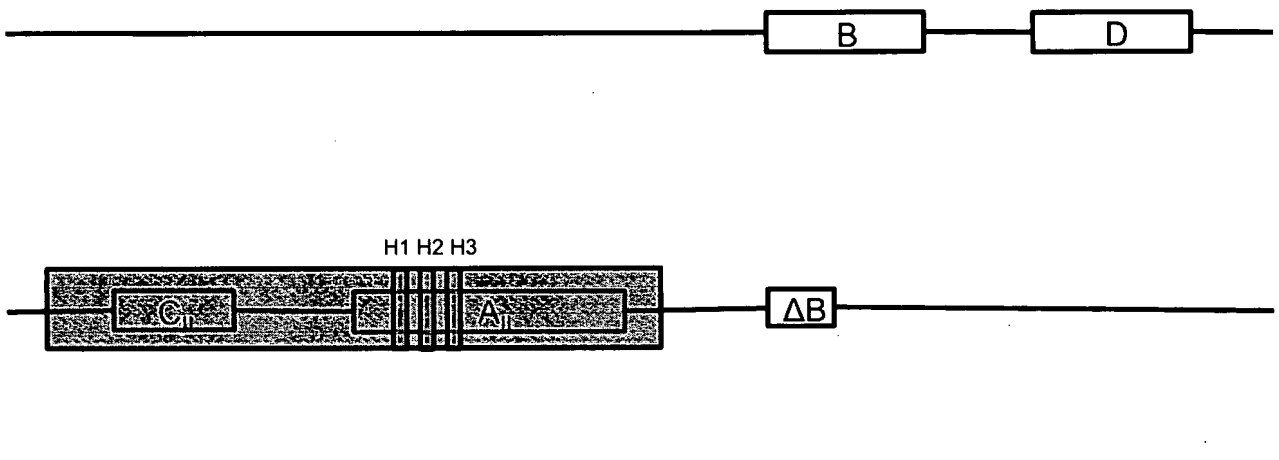
ApxIA is not secreted

No exotoxin secretion

Immunogenicity: None
Hemolysis : None

Prideaux *et al.*, US 6,472,183

3) Inactivation or deletion of *apxIIC* gene (activation gene) and *apxIIA* gene (structural exotoxin gene) of wild strain HS93 (Serovar 7): strain Tox$^-$ (Example 5)



Operon *apxICABD* *apxIC* and *apxIA* genes naturally inactivated

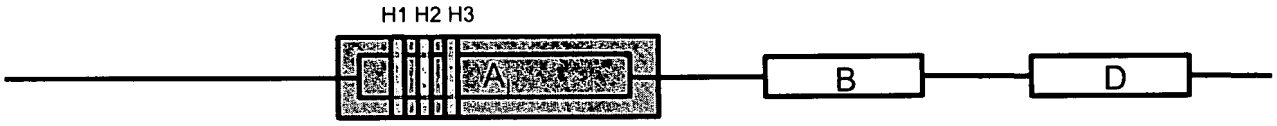
Operon *apxIICAΔB* *apxIIC* and *apxIIA* genes inactivated or deleted

No secretion of exotoxins:

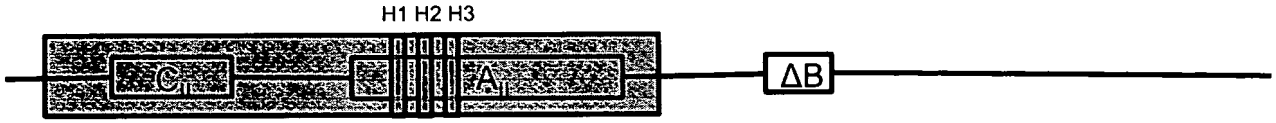
- ApxI exotoxin is naturally not produced
- ApxII exotoxin is not produced because of inactivation of *apxIIC* and *apxIIA* genes

Prideaux *et al.*, US 6,472,183

4) Insertion of *apxIA* gene (structural exotoxin gene) in strain Tox$^-$:
strain Tox$^-$/pI63B-TIK
(Examples 3,4, 5 and 6; column 4, lines 58-65)



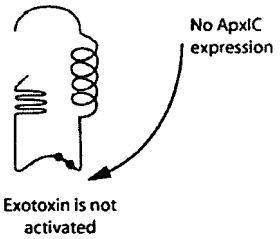
Operon *apxICABD*
apxIC gene naturally inactivated
apxIA gene inserted

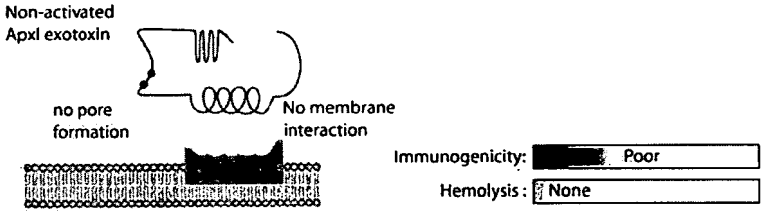


Operon *apxIICAΔB*
apxIIC and *apxIIA* genes inactivated ordeleted

Production of non-activated ApxI exotoxin because *apxIC* gene is naturally inactivated
No production of ApxII exotoxin because *apxIIC* and *apxIIA* genes are inactivated
(see Figure IV)

FIGURE IV
apxIA and not *apxIC* genes
or
apxIIA and Δ*apxIIC* genes
(Prideaux)





Conclusions

Pinol *et al.*, US 2006/0051371-A1

1.- Technical concept
Mutation (deletion) in a transmembrane domain of exotoxin A genes

2.- Novelty
None of the documents of the state of the art discloses a mutation (deletion) in a transmembrane domain of *apxIA* gene, with or without a mutation (deletion) in a transmembrane domain of *apxIIA* gene.

3.- Inventive step
Once a mutation (deletion) in a transmembrane domain of *apxIA* gene or in a transmembrane domains *of apxIA* and *apxIIA* genes has been performed, it would not have been obvious for the skilled person that the protein:

a) would maintain the structure
b) would be secreted
c) would not be haemolytic
d) would be immunogenic
e) would be immunoprotective

Pinol *et al.*, US 2006/0051371-A1

4.- Applicant strains are highly immunogenic and non-haemolytic because:

a) they produce and secrete activated ApxI and ApxII exotoxins

b) these exotoxins are not capable of forming pores

5.- So, a mutation (deletion) carried out in a transmembrane domain of the *apxIA* gene, with or without a mutation (deletion) in a transmembrane domain of the *apxIIA* gene surprisingly resulted in:

- maintenance of the structure of ApxI and ApxII exotoxins,
- secretion of the ApxI and ApxII exotoxins,
- non-haemolytic activity,
- immunogenicity and
- immunoprotective characteristics

6.- Claims 13, 14, 15, 16, 17 and 19 currently on file are drawn to immunogenic, non-haemolytic APP strains comprising at least a mutation (deletion) in a transmembrane domain region of the *apxIA* gene, and optionally, a mutation in a transmembrane domain region of the *apxIIA* gene.

7.- Any of the documents cited in the prior art do not disclose, suggest or teach APP strains obtained by mutation (deletion) in a segment of the transmembrane domain region of the *apxIA* gene, with or without a mutation (deletion) in a segment of the transmembrane domain region of the *apxIIA* gene.

8.- All documents cited in the prior art were driven by the same idea and purpose: that the absence of the main virulence factor of APP, i.e. Apx toxins, (by deletion, or non-activation, or non-secretion) would result in a non-virulent (non-haemolytic), but protective strain.

Pinol *et al.*, US 2006/0051371-A1

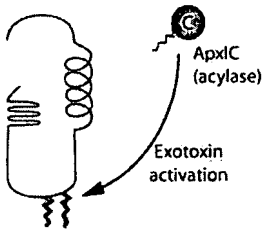
9.- In APP this strategy resulted less efficient than in other microorganisms, because Apx toxins need to be activated and secreted in order to induce a high level of immunoprotection.

10.- It would not have been obvious for the skilled person that a mutation (deletion) in a transmembrane domain region of the *apxIA* gene, with or without a mutation (deletion) in a transmembrane domain region of the *apxIIA* gene would lead to an APP strain expressing and secreting activated Apx toxins, so maintaining its immunogenic properties, but not its haemolytic activity, resulting consequently in a non-virulent strain being not capable of producing pores in target cells.

Illustrated summary with idealized structures and mechanisms

(without being bound to the theory)

FIGURE I
apxIA and *apxIC* genes
(Reimer et al.)



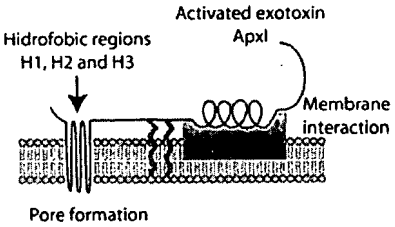
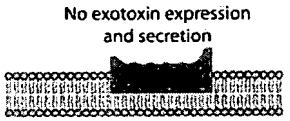


FIGURE II
ΔapxICABD genes
(Reimer et al.)



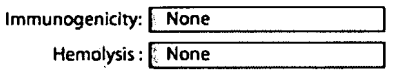
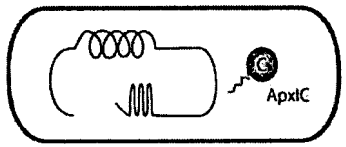
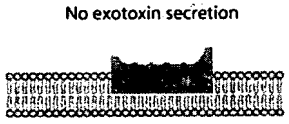


FIGURE III
apxIA and *apxIC* genes
ΔapxIB and *ΔapxID* genes
(MacInnes et al.)
(Prideaux et al.)





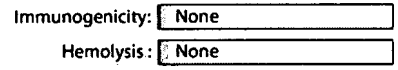
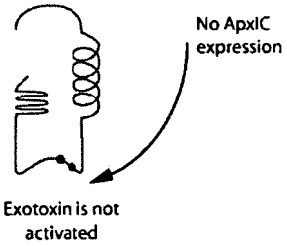
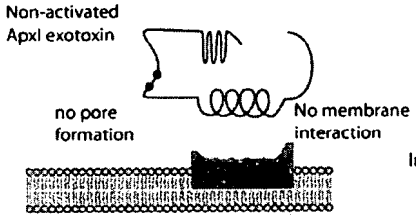


FIGURE IV
apxIA and not *apxIC* genes
or
apxIIA and *ΔapxIIC* genes
(Prideaux et al.)





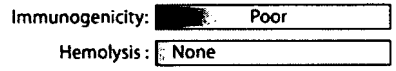


FIGURE V
apxIAΔH2 + *apxIC* genes
(HIPRA 1)